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Dated: 6-25-08 Signature: Pamela Harrison  
(Pamela Harrison)

Docket No.: 104831-0002-103  
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:  
Chang et al.

Confirmation No.: 9375

Application No.: 10/657383

Art Unit: 1623

Filed: September 8, 2003

Examiner: Maier, Leigh C.

For: METHOD FOR ENHANCING THE  
EFFECTIVENESS OF CANCER THERAPIES

MS Amendment  
Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

**Declaration Under 37 C.F.R. § 1.131 of Joseph Grimm**

Sir:

I, Joseph Grimm of hereby declare as follows:

1. I am the President of Prospect Therapeutics, Inc. ("Prospect"), which is the Assignee of the entire right, title and interest in the instant application. A Statement Under 37 C.F.R. § 3.73(b) establishing Prospect's ownership was submitted on May 22, 2007.
2. A Petition Under 37 C.F.R. § 1.47 was submitted to the Office on May 25, 2007 and a Request for Reconsideration, which included a declaration executed by me, was submitted on October 1, 2007. The Office granted the Petition Under 37 C.F.R. § 1.47 on November 7, 2007.
3. On information and belief, the inventors completed the invention as described and claimed in the above-identified application prior to March 27, 2001.
4. In support of this, I include herewith as Exhibit A a protocol design for a study, which I believe to have been carried out at the inventors' direction, designed to test the efficacy of interferon- $\alpha$ 2b (IFN- $\alpha$ 2b), GBC590B, and combinations thereof in a pancreatic carcinoma xenograft mouse model. IFN- $\alpha$ 2b is an oncolytic cytokine, and GBC590B is a modified pectin that comprises a polymeric backbone having side chains terminated by galactose or arabinose units.

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5. Exhibit B shows the results of this study. As can be seen, at the end of one week, the tumor size in all groups averaged 113-114 mg. However, as the experiment progressed, the average tumor size in groups receiving both GBC590B and interferon consistently lagged behind that of those receiving IFN or GBC-590 alone. By Day 18, the last date when all animals in these groups still survived, the mice receiving only IFN (Group 3) had tumors averaging 958.7 mg, while those receiving IFN with GBC-590 had tumors averaging 916.6 mg, 832.5 mg, and 906.9 mg, indicating that tumor growth was slower in these groups. At subsequent measurement times, after the death of some of the mice, the disparity increased dramatically, indicating that the combined therapy was particularly effective in slowing tumor growth in some of the mice. As then summarized in Exhibit C, administration of either therapy alone was insufficient to achieve a significant improvement in the lifespan (MDS, mean day of survival) of the test mice (i.e., the difference was within the margins of error), and no mice survived to the end of the experiment. In contrast, a combination of the therapies resulted in survival of some of the test mice, and in fact the combination allowed a lower dose of IFN- $\alpha$ 2b to be used efficaciously. Indeed, two mice survived at lower doses of IFN- $\alpha$ 2b (Groups 5 and 6) than at the dose that was, by itself, unable to achieve any significant benefit (Groups 3 and 4). Although the MDS does not show improvement, this number is calculated excluding the mice that survived (20% of the total test mice for groups 5 and 6). Accordingly, the results demonstrate that GBC590B enhances the efficacy of IFN- $\alpha$ 2b, and in particular, enhances its ability to inhibit tumor growth.

6. By the time of the study described above, it was generally known in the art that modified pectin binds galectins, such as galectin-3, through its galactose residues and that other galectin-binding carbohydrates would be expected to have similar biological activities. For example, an article by Platt (an undersigned co-inventor of the instant application) and Raz ("Modulation of the Lung Colonization of B16-F1 Melanoma Cells by Citrus Pectin," *Journal of the National Cancer Institute*, 84: 438-442 (1992), Exhibit D) discusses a prior study showing that galactoside-binding lectins have been shown to mediate cell-cell adhesion and cell-extracellular matrix adhesion through carbohydrates containing terminal galactosyl residues. The article reports another prior study that liver metastasis of murine L-1 sarcoma cells was inhibited by D-galactose and arabinogalactan. Based upon this prior work, the article evaluates molecules rich in galactoside residues for modulating tumor cell colonization *in vivo*. In addition, U.S. Patent No. 5,834,442 (Exhibit E), filed July 7, 1994 and issued November 10, 1998, states that it had been previously demonstrated that modified citrus pectin could interfere with cell-cell interactions mediated by cell surface carbohydrate-binding galectin-3 molecules. This patent then teaches that complex carbohydrates rich in galactoside residues, such as pectin, act as potent inhibitors of prostate carcinoma metastasis. Furthermore, U.S. Patent No. 5,681,923 (Exhibit F), filed October 6, 1995 and issued October 28, 1997, for which co-inventor Platt is the sole inventor, discloses the sequence of galactose-specific binding polypeptides and the description of Figure 1 teaches that galactose bound to such polypeptides can be a simple sugar or a portion of a polysaccharide. Based on the inventors' knowledge of these facts and the results described in paragraphs 3 and 4, it is my belief that the inventors expected that galectin-binding carbohydrates generally, particularly those containing terminal galactose moieties, would be useful in the invention.

7. To the best of my knowledge, the results described in paragraph 4 were obtained in the United States through experiments performed by the inventors in collaboration with researchers

working under the inventors' direction, and were obtained in a report dated prior to March 27, 2001. The dates redacted from Exhibit B are all prior to March 27, 2001.

8. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title XVIII of the United States Code and that willful false statements may jeopardize the validity of this Application for Patent or any patent issuing thereon.

Joseph Grimm, President,  
Prospect Therapeutics, Inc.

Dated: 5/27/08

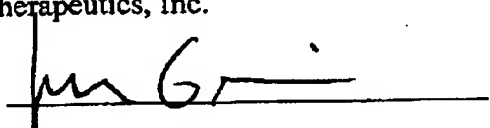
Signature: 

Table 1

Protocol Design for the Panc-e20 Study

Group	n	Treatment Regimen 1				Treatment Regimen 2			
		Agent	mg/kg	Route	Schedule	Agent	mg/kg	Route	Schedule
1	10	Vehicle	—	iv	D1,2,4,6,8,10,12,14	—	—	—	—
2	10	GBC590B	6.4	iv	D1,2,4,6,8,10,12,14	—	—	—	—
3	10	IFN- $\alpha$ 2b	10 x 10 <sup>6</sup> Units/kg	sc	qd x 14	—	—	—	—
4	10	GBC590B	6.4	iv	D1,2,4,6,8,10,12,14	IFN- $\alpha$ 2b	10 x 10 <sup>6</sup> Units/kg	sc	qd x 14
5	10	GBC590B	6.4	iv	D1,2,4,6,8,10,12,14	IFN- $\alpha$ 2b	5 x 10 <sup>6</sup> Units/kg	sc	qd x 14
6	10	GBC590B	6.4	iv	D1,2,4,6,8,10,12,14	IFN- $\alpha$ 2b	2.5 x 10 <sup>6</sup> Units/kg	sc	qd x 14

[illegible]

**Group 2: GBCS90 (6.4 mg/kg)**

Year	1900	1901	1902	1903	1904	1905	1906	1907	1908	1909	1910	1911	1912	1913	1914	1915	1916	1917	1918	1919	1920	1921	1922	1923	1924	1925	1926	1927	1928	1929	1930	1931	1932	1933	1934	1935	1936	1937	1938	1939	1940	1941	1942	1943	1944	1945	1946	1947	1948	1949	1950	1951	1952	1953	1954	1955	1956	1957	1958	1959	1960	1961	1962	1963	1964	1965	1966	1967	1968	1969	1970	1971	1972	1973	1974	1975	1976	1977	1978	1979	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100
1900	1901	1902	1903	1904	1905	1906	1907	1908	1909	1910	1911	1912	1913	1914	1915	1916	1917	1918	1919	1920	1921	1922	1923	1924	1925	1926	1927	1928	1929	1930	1931	1932	1933	1934	1935	1936	1937	1938	1939	1940	1941	1942	1943	1944	1945	1946	1947	1948	1949	1950	1951	1952	1953	1954	1955	1956	1957	1958	1959	1960	1961	1962	1963	1964	1965	1966	1967	1968	1969	1970	1971	1972	1973	1974	1975	1976	1977	1978	1979	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100	

Group 3: 1PM-4PM (10:10-11:20 AM)

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	Day 16	Day 17	Day 18	Day 19	Day 20	Day 21	Day 22	Day 23	Day 24	Day 25	Day 26	Day 27	Day 28	Day 29	Day 30	Day 31	Day 32	Day 33	Day 34	Day 35	Day 36	Day 37	Day 38	Day 39	Day 40	Day 41	Day 42	Day 43	Day 44	Day 45	Day 46	Day 47	Day 48	Day 49	Day 50	Day 51	Day 52	Day 53	Day 54	Day 55	Day 56	Day 57	Day 58	Day 59	Day 60	Day 61	Day 62	Day 63	Day 64	Day 65	Day 66	Day 67	Day 68	Day 69	Day 70	Day 71	Day 72	Day 73	Day 74	Day 75	Day 76	Day 77	Day 78	Day 79	Day 80	Day 81	Day 82	Day 83	Day 84	Day 85	Day 86	Day 87	Day 88	Day 89	Day 90	Day 91	Day 92	Day 93	Day 94	Day 95	Day 96	Day 97	Day 98	Day 99	Day 100	Day 101	Day 102	Day 103	Day 104	Day 105	Day 106	Day 107	Day 108	Day 109	Day 110	Day 111	Day 112	Day 113	Day 114	Day 115	Day 116	Day 117	Day 118	Day 119	Day 120	Day 121	Day 122	Day 123	Day 124	Day 125	Day 126	Day 127	Day 128	Day 129	Day 130	Day 131	Day 132	Day 133	Day 134	Day 135	Day 136	Day 137	Day 138	Day 139	Day 140	Day 141	Day 142	Day 143	Day 144	Day 145	Day 146	Day 147	Day 148	Day 149	Day 150	Day 151	Day 152	Day 153	Day 154	Day 155	Day 156	Day 157	Day 158	Day 159	Day 160	Day 161	Day 162	Day 163	Day 164	Day 165	Day 166	Day 167	Day 168	Day 169	Day 170	Day 171	Day 172	Day 173	Day 174	Day 175	Day 176	Day 177	Day 178	Day 179	Day 180	Day 181	Day 182	Day 183	Day 184	Day 185	Day 186	Day 187	Day 188	Day 189	Day 190	Day 191	Day 192	Day 193	Day 194	Day 195	Day 196	Day 197	Day 198	Day 199	Day 200	Day 201	Day 202	Day 203	Day 204	Day 205	Day 206	Day 207	Day 208	Day 209	Day 210	Day 211	Day 212	Day 213	Day 214	Day 215	Day 216	Day 217	Day 218	Day 219	Day 220	Day 221	Day 222	Day 223	Day 224	Day 225	Day 226	Day 227	Day 228	Day 229	Day 230	Day 231	Day 232	Day 233	Day 234	Day 235	Day 236	Day 237	Day 238	Day 239	Day 240	Day 241	Day 242	Day 243	Day 244	Day 245	Day 246	Day 247	Day 248	Day 249	Day 250	Day 251	Day 252	Day 253	Day 254	Day 255	Day 256	Day 257	Day 258	Day 259	Day 260	Day 261	Day 262	Day 263	Day 264	Day 265	Day 266	Day 267	Day 268	Day 269	Day 270	Day 271	Day 272	Day 273	Day 274	Day 275	Day 276	Day 277	Day 278	Day 279	Day 280	Day 281	Day 282	Day 283	Day 284	Day 285	Day 286	Day 287	Day 288	Day 289	Day 290	Day 291	Day 292	Day 293	Day 294	Day 295	Day 296	Day 297	Day 298	Day 299	Day 300	Day 301	Day 302	Day 303	Day 304	Day 305	Day 306	Day 307	Day 308	Day 309	Day 310	Day 311	Day 312	Day 313	Day 314	Day 315	Day 316	Day 317	Day 318	Day 319	Day 320	Day 321	Day 322	Day 323	Day 324	Day 325	Day 326	Day 327	Day 328	Day 329	Day 330	Day 331	Day 332	Day 333	Day 334	Day 335	Day 336	Day 337	Day 338	Day 339	Day 340	Day 341	Day 342	Day 343	Day 344	Day 345	Day 346	Day 347	Day 348	Day 349	Day 350	Day 351	Day 352	Day 353	Day 354	Day 355	Day 356	Day 357	Day 358	Day 359	Day 360	Day 361	Day 362	Day 363	Day 364	Day 365	Day 366	Day 367	Day 368	Day 369	Day 370	Day 371	Day 372	Day 373	Day 374	Day 375	Day 376	Day 377	Day 378	Day 379	Day 380	Day 381	Day 382	Day 383	Day 384	Day 385	Day 386	Day 387	Day 388	Day 389	Day 390	Day 391	Day 392	Day 393	Day 394	Day 395	Day 396	Day 397	Day 398	Day 399	Day 400	Day 401	Day 402	Day 403	Day 404	Day 405	Day 406	Day 407	Day 408	Day 409	Day 410	Day 411	Day 412	Day 413	Day 414	Day 415	Day 416	Day 417	Day 418	Day 419	Day 420	Day 421	Day 422	Day 423	Day 424	Day 425	Day 426	Day 427	Day 428	Day 429	Day 430	Day 431	Day 432	Day 433	Day 434	Day 435	Day 436	Day 437	Day 438	Day 439	Day 440	Day 441	Day 442	Day 443	Day 444	Day 445	Day 446	Day 447	Day 448	Day 449	Day 450	Day 451	Day 452	Day 453	Day 454	Day 455	Day 456	Day 457	Day 458	Day 459	Day 460	Day 461	Day 462	Day 463	Day 464	Day 465	Day 466	Day 467	Day 468	Day 469	Day 470	Day 471	Day 472	Day 473	Day 474	Day 475	Day 476	Day 477	Day 478	Day 479	Day 480	Day 481	Day 482	Day 483	Day 484	Day 485	Day 486	Day 487	Day 488	Day 489	Day 490	Day 491	Day 492	Day 493	Day 494	Day 495	Day 496	Day 497	Day 498	Day 499	Day 500	Day 501	Day 502	Day 503	Day 504	Day 505	Day 506	Day 507	Day 508	Day 509	Day 510	Day 511	Day 512	Day 513	Day 514	Day 515	Day 516	Day 517	Day 518	Day 519	Day 520	Day 521	Day 522	Day 523	Day 524	Day 525	Day 526	Day 527	Day 528	Day 529	Day 530	Day 531	Day 532	Day 533	Day 534	Day 535	Day 536	Day 537	Day 538	Day 539	Day 540	Day 541	Day 542	Day 543	Day 544	Day 545	Day 546	Day 547	Day 548	Day 549	Day 550	Day 551	Day 552	Day 553	Day 554	Day 555	Day 556	Day 557	Day 558	Day 559	Day 560	Day 561	Day 562	Day 563	Day 564	Day 565	Day 566	Day 567	Day 568	Day 569	Day 570	Day 571	Day 572	Day 573	Day 574	Day 575	Day 576	Day 577	Day 578	Day 579	Day 580	Day 581	Day 582	Day 583	Day 584	Day 585	Day 586	Day 587	Day 588	Day 589	Day 590	Day 591	Day 592	Day 593	Day 594	Day 595	Day 596	Day 597	Day 598	Day 599	Day 600	Day 601	Day 602	Day 603	Day 604	Day 605	Day 606	Day 607	Day 608	Day 609	Day 610	Day 611	Day 612	Day 613	Day 614	Day 615	Day 616	Day 617	Day 618	Day 619	Day 620	Day 621	Day 622	Day 623	Day 624	Day 625	Day 626	Day 627	Day 628	Day 629	Day 630	Day 631	Day 632	Day 633	Day 634	Day 635	Day 636	Day 637	Day 638	Day 639	Day 640	Day 641	Day 642	Day 643	Day 644	Day 645	Day 646	Day 647	Day 648	Day 649	Day 650	Day 651	Day 652	Day 653	Day 654	Day 655	Day 656	Day 657	Day 658	Day 659	Day 660	Day 661	Day 662	Day 663	Day 664	Day 665	Day 666	Day 667	Day 668	Day 669	Day 670	Day 671	Day 672	Day 673	Day 674	Day 675	Day 676	Day 677	Day 678	Day 679	Day 680	Day 681	Day 682	Day 683	Day 684	Day 685	Day 686	Day 687	Day 688	Day 689	Day 690	Day 691	Day 692	Day 693	Day 694	Day 695	Day 696	Day 697	Day 698	Day 699	Day 700	Day 701	Day 702	Day 703	Day 704	Day 705	Day 706	Day 707	Day 708	Day 709	Day 710	Day 711	Day 712	Day 713	Day 714	Day 715	Day 716	Day 717	Day 718	Day 719	Day 720	Day 721	Day 722	Day 723	Day 724	Day 725	Day 726	Day 727	Day 728	Day 729	Day 730	Day 731	Day 732	Day 733	Day 734	Day 735	Day 736	Day 737	Day 738	Day 739	Day 740	Day 741	Day 742	Day 743	Day 744	Day 745	Day 746	Day 747	Day 748	Day 749	Day 750	Day 751	Day 752	Day 753	Day 754	Day 755	Day 756	Day 757	Day 758	Day 759	Day 760	Day 761	Day 762	Day 763	Day 764	Day 765	Day 766	Day 767	Day 768	Day 769	Day 770	Day 771	Day 772	Day 773	Day 774	Day 775	Day 776	Day 777	Day 778	Day 779	Day 780	Day 781	Day 782	Day 783	Day 784	Day 785	Day 786	Day 787	Day 788	Day 789	Day 790	Day 791	Day 792	Day 793	Day 794	Day 795	Day 796	Day 797	Day 798	Day 799	Day 800	Day 801	Day 802	Day 803	Day 804	Day 805	Day 806	Day 807	Day 808	Day 809	Day 810	Day 811	Day 812	Day 813	Day 814	Day 815	Day 816	Day 817	Day 818	Day 819	Day 820	Day 821	Day 822	Day 823	Day 824	Day 825	Day 826	Day 827	Day 828	Day 829	Day 830	Day 831	Day 832	Day 833	Day 834	Day 835	Day 836	Day 837	Day 838	Day 839	Day 840	Day 841	Day 842	Day 843	Day 844	Day 845	Day 846	Day 847	Day 848	Day 849	Day 850	Day 851	Day 852	Day 853	Day 854	Day 855	Day 856	Day 857	Day 858	Day 859	Day 860	Day 861	Day 862	Day 863	Day 864	Day 865	Day 866	Day 867	Day 868	Day 869	Day 870	Day 871	Day 872	Day 873	Day 874	Day 875	Day 876	Day 877	Day 878	Day 879	Day 880	Day 881	Day 882	Day 883	Day 884	Day 885	Day 886	Day 887	Day 888	Day 889	Day 890	Day 891	Day 892	Day 893	Day 894	Day 895	Day 896	Day 897	Day 898	Day 899	Day 900	Day 901	Day 902	Day 903	Day 904	Day 905	Day 906	Day 907	Day 908	Day 909	Day 910	Day 911	Day 912	Day 913	Day 914	Day 915	Day 916	Day 917	Day 918	Day 919	Day 920	Day 921	Day 922	Day 923	Day 924	Day 925	Day 926	Day 927	Day 928	Day 929	Day 930	Day 931	Day 932	Day 933	Day 934	Day 935	Day 936	Day 937	Day 938	Day 939	Day 940	Day 941	Day 942	Day 943	Day 944	Day 945	Day 946	Day 947	Day 948	Day 949	Day 950	Day 951	Day 952	Day 953	Day 954	Day 955	Day 956	Day 957	Day 958	Day 959	Day 960	Day 961	Day 962	Day 963	Day 964	Day 965	Day 966	Day 967	Day 968	Day 969	Day 970	Day 971	Day 972	Day 973	Day 974	Day 975	Day 976	Day 977	Day 978	Day 979	Day 980	Day 981	Day 982	Day 983	Day 984	Day 985	Day 986	Day 987	Day 988	Day 989	Day 990	Day 991	Day 992	Day 993	Day 994	Day 995	Day 996	Day 997	Day 998	Day 999	Day 1000
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Group 54 CAC 350 is assigned and FN-100 is (not) assigned

[illegible]

**Table 2**  
**Response Summary for the Panc-e20 Study**

Group	n	Regimen 1		Regimen 2		MDS to 1.2 g ± SEM (n)	# Toxic Deaths	# of Survivors	# CR	# PR	# Stable Disease
		Agent	mg/kg	Agent	mg/kg						
1	10	Vehicle	---	---	---	22.6 ± 1.9 (9)	1*	0	0	0	0
2	10	GBCS90B	6.4	---	---	23.0 ± 2.4 (10)	0	0	0	0	0
3	10	IFN-α2b 10 x 10 <sup>4</sup> Units/kg	6.4	---	---	21.9 ± 1.8 (10)	0	0	0	0	0
4	10	GBCS90B	6.4	IFN-α2b 10 x 10 <sup>6</sup> Units/kg	10 x 10 <sup>6</sup> Units/kg	20.9 ± 1.6 (9)	0	1	1	0	0
5	10	GBCS90B	6.4	IFN-α2b 5 x 10 <sup>6</sup> Units/kg	5 x 10 <sup>6</sup> Units/kg	20.1 ± 1.0 (8)	0	2	2	0	0
6	10	GBCS90B	6.4	IFN-α2b 2.5 x 10 <sup>6</sup> Units/kg	2.5 x 10 <sup>6</sup> Units/kg	20.3 ± 2.0 (8)	0	2	2	0	0

\*The mouse escaped and was euthanized.

Table 3. Toxic effects of MST-16 therapy

Toxic effect	No. of patients (%)	Toxicity grade			
		1	2	3	4
Leukopenia	19 (70)		4	7	8
Anemia	14 (52)	1	3	8	2
Thrombocytopenia	12 (44)	2		5	3
Elevation of aspartate aminotransferase/ alanine aminotransferase	4 (15)	1	3		
Elevation of total bilirubin level	1 (4)		1		
Nausea/vomiting	10 (37)	7	3		
Diarrhea	10 (37)	2	6	2	
Stomatitis	9 (33)	4	5		
Soreness	5 (19)	1	2	1	1
Alopecia	3 (7)	1	1		
Pyrexia	1 (4)	1			

Furthermore, studies of combination chemotherapy with other antitumor drugs are warranted, since, in Japan, MST-16 has been shown to have antitumor activity in combination with other drugs in vitro and in vivo. These studies have demonstrated supra-additive effects on in vitro growth of MOLT-3 cells when the drug was used in combination with doxorubicin, amstarine, and bleomycin, as well as additive effects with cyclophosphamide, cisplatin, mitomycin-C, and cytarabine (19). MST-16 has also had supra-additive effects on L1210 leukemia in mice in combination with doxorubicin, mitomycin-C, cisplatin, cyclophosphamide, and cytarabine (20). In addition, it is being used against breast cancer, gastric cancer, and adult T-cell leukemia/lymphoma in phase II trials in Japan.

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## Modulation of the Lung Colonization of B16-F1 Melanoma Cells by Citrus Pectin

David Plan; Avraham Raz\*

*Context:* Studies have shown that the galactoside-containing simple sugars and anti-galactoside-binding lectin antibodies may affect experimental tumor cell metastasis. However, the limited number of reagents used thus far necessitate further observations. *Purpose:* Natural citrus pectin (CP) and pH-modified CP (MCP), rich in galactose residues, were used to study the involvement of carbohydrates containing galactoside residues in cellular interaction in vitro and in lung colonization in vivo of B16-F1 melanoma cells. *Methods:* B16-F1 melanoma cells were incubated with various concentrations of CP and MCP. Their ability to form homotypic aggregation in vitro and tumor lung colonization in vivo in 8-week-old female C57BL/6 mice was then analyzed. *Results:* The CP binds to the surface of B16-F1 melanoma cells; this binding can be inhibited by lactose at a concentration of 0.15 M. Intravenous injection of the murine B16-F1 melanoma cells with the natural CP resulted in a significant increase (up to threefold) in the appearance of tumor colonies in the lung and in increased homotypic aggregation properties of the cells, while injection of MCP significantly decreased B16-F1 experimental metastasis (>90%). *Conclusions:* Tumor galactoside-binding proteins mediate cellular recognition by linking oligosaccharides with terminal D-galactoside residues on adjacent cells. Successful interference



with such a process with MCP may lead to a reduced ability to form tumor cell emboli and metastasis. **Implications:** These findings imply that the galactose-containing carbohydrate side chains of CP might mimic or compete with the natural ligand(s) of the tumor galactoside-binding protein (gal-lectin) and thus affect cellular interactions relevant for metastasis. [J Natl Cancer Inst 84:438-442, 1992]

Previously, galactoside-binding lectins were shown to mediate cell-cell adhesion and cell-extracellular matrix adhesion through carbohydrates containing terminal or penultimate galactosyl residues. The role of galactose residues and their complementary receptors in this process was previously demonstrated, leading to the exploration of their possible use for the understanding of and intervention in tumor metastasis (1,2). Experimental liver metastasis of the murine L-1 sarcoma cells was inhibited by D-galactose and arabinogalactan (3), while methyl- $\alpha$ -D-lactoside and lacto-N-tetrose caused significant reduction in the metastatic deposition of B16 melanoma cells compared with the control (4). Treatment of B16 melanoma and UV-2237 fibrosarcoma cells in vitro with monoclonal antibody directed against tumor galactoside-binding protein (gal-lectin) before their injection into the tail veins of syngeneic mice resulted in a marked decrease in the development of tumor colonies in the lung (5). Furthermore, a correlation was established between the level of a human gal-lectin and the serum level of carcino-embryonic antigen and the stage of progression of colorectal carcinoma in human patients. This correlation suggests

a role for gal-lectin in human colon cancer (6).

In this investigation, we have used natural citrus pectin (CP) and pH-modified CP (MCP), molecules which are rich in galactoside residues, to further evaluate the possible use of carbohydrate-containing galactosyl residues for augmenting tumor cell colonization in vivo.

## Materials and Methods

### CP and Its Modification

CP (70-100 kd; 0.5%; Sigma Chemical Co., St. Louis, Mo.; 10% methoxyl groups) was solubilized and sterilized under UV radiation for 48 hours. The total carbohydrate level was determined by the phenol sulfuric acid method (7). The pH of CP was modified by increasing the pH to 10.0 with NaOH (3 N) for 30 minutes and then by decreasing it to 3.0 with HCl (3 N) according to the method of Albersheim et al. (8). Samples were taken after 10 hours and 24 hours, and the pH of the samples was equilibrated to 6.3. The solutions were washed with ethanol (70%) and dried with acetone (100%), resulting in MCP fragments of approximately 10 kd. A sample of dried MCP was rehydrated with  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free phosphate-buffered saline (pH 7.2) (CMF-PBS) to a final stock solution of 0.5% (w/vol). The molecular weights of CP and MCP were determined by viscosity measurements (9) at 25 °C in an Ubbelohde No. 1 viscometer (Ubbelohde, The Netherlands) with sodium-hexametaphosphate at 20 mM (pH 4.5), 0.2% EDTA, and (0.9%) NaCl.

Natural sugars in CP were estimated from the difference between the *m*-hydroxyphenol method (10) and the total carbohydrates with phenol sulfuric acid (7). The composition of the natural sugars was obtained by hydrolysis in trifluoroacetic acid (2 N). The respective alditol acetates were analyzed by gas-liquid chromatography as described (8,11,12). CP was radiolabeled by oxidation with  $\text{NaIO}_4$ , followed by reduction with  $\text{NaBH}_4$  (13).

### Cells and Culture Conditions

B16-F1 melanoma cells (13) were grown in Dulbecco's modified Eagle medium (GIBCO Laboratories, Inc., Grand Island, N.Y.) containing 10% heat-inactivated

fetal bovine serum, nonessential amino acids, and antibiotics. Cell cultures were incubated in a humidifier atmosphere of 7%  $\text{CO}_2$  and 93% air. To ensure reproducibility, all experiments were performed with cultures grown for no longer than 6 weeks after recovery from frozen stocks.

### Lung Colonization Assay

B16-F1 cells grown to 70% confluence were detached with 2 mM EDTA in CMF-PBS. The cells were then washed and resuspended in CMF-PBS with or without CP and MCP, and aliquots of the suspension containing  $1 \times 10^5$  cells in 0.2 mL were injected intravenously into the tail veins of 8-week-old female C57BL/6 mice. After 17 days, the mice were autopsied. The number of tumor colonies in the lung was determined under a dissecting microscope (14).

### Assay for CP-Induced Homotypic Aggregation

Cells were detached with 2 mM EDTA in CMF-PBS and suspended at  $1 \times 10^5$  cells/mL in CMF-PBS as described (7) with and without 0.05% CP or 0.05% MCP. Aliquots containing 0.5 mL of cell suspension were placed in siliconized glass tubes and agitated at 50 rpm for 30 minutes at 37 °C. The aggregation was then terminated by fixing the cells with 1% formaldehyde in CMF-PBS. Samples were used for counting the number of single cells, and aggregation was calculated according to the following equation:

$$(1 - N_i/N_c) \times 100,$$

where  $N_i$  and  $N_c$  represent the number of single cells in the presence of the tested compounds and the number of single cells in the control buffer (CMF-PBS), respectively.

## Results and Discussion

The lodgment, attachment, and growth of blood-borne neoplastic cells depend largely on cell embolization. The arrest of intravenously inoculated aggregates of tumor cells leading to intense metastatic growth is much higher than that of single cells. Furthermore, several studies using the same B16-F1 melanoma cell system have demonstrated a correlation between the tendency of the cells to undergo inter-

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Cancer Metastasis Program, Michigan Cancer Foundation, Detroit, Mich.

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Correspondence to: Avram Raz, Ph.D., Metastasis Research Program, Michigan Cancer Foundation, 110 E. Warren Ave., Detroit, MI 48201-1379.

cellular interactions in culture and their metastatic potential. Previously, we (1) suggested a molecular basis for such processes and demonstrated that several tumor cells, including the B16-F1 melanoma cells, contain galactoside-binding lectin which mediates cell homotypic aggregation in the presence of the asialoglycoproteins. Carbohydrates containing galactoside residues and antibodies directed against the gal-lectin were shown to reduce the tendency of tumor cells to develop metastases (1-5). The effect of CP on such processes was tested in the search for additional reagents for evaluation of the possible relationship between the gal-lectin and the endogenous ligand.

CP is a branched complex polysaccharide polymer responsible for the texture of fruits and vegetables. The CPs consist of partially esterified galacturonic acid residues with side chains composed of arabinose, galactose, glucose, mannose, and xylose. The sugar composition of CP would indicate that the anhydrogalacturonic acid comprises about 50% of the total residues, while galactose and arabinose constitute the two other major carbohydrates of CP, comprising 20% and 15%, respectively (Fig. 1). The modification of CP to MCP by pH involves degradation of the main galacturonic acid chain by  $\beta$ -elimination (high pH) followed by partial degradation of the natural carbohydrates (low pH), resulting in nonbranched carbohydrate chains of basically the same sugar composition of the unmodified CP (8,15).

The B16-F1 melanoma cells exhibited a low level of spontaneous homotypic aggregation, clearing a 1-hour agitation in CMF-PBS (Fig. 2, A). The aggregation of the cells, however, was markedly increased in the presence of 0.05% CP (Fig. 2, A). In contrast, an equal concentration of the nonbranched MCP failed to stimulate cell aggregation (Fig. 2, A). It is conceivable that the cell-surface gal-lectins recognize and bind galactosyl residues on different side chains of the same CP molecules, which serves as a cross-linking bridge between cells and subsequently leads to the formation of cell aggregates, while the nonbranched MCP fails to cross-link. The pectin used here is a structural cell wall polysaccharide present in all higher plants. It is primarily a polymer of D-galacturonic acid. The

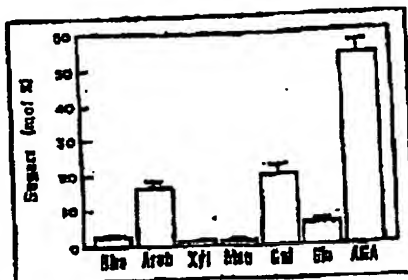


Fig. 1. Sugar composition of CP (mol %)—10% methoxyl group. The amount of galacturonic acid (AGA) was determined according to the method reported in (10), and total carbohydrate was ascertained by phenol sulfuric acid reaction according to the technique reported in (7). Total natural sugars were calculated from the difference between the two reactions based on galacturonic acid and glucose (Glu) standards. The composition and the amounts of individual natural sugars were obtained by hydrolysis in trifluoroacetic acid (2 M). The respective aldol sequences were analyzed by gas chromatography according to the method reported in (11). Ara = arabinose; Xyl = xylose; Man = mannose; Gal = galactose.

structural unit of all pectin molecules is a linear chain of (1-4)-linked  $\alpha$ -D-galactopyranosyluronic acid (8,13-16). Further clarification of the nature of the interaction between the cells and CP came from studies that demonstrated a complete inhibition of [ $^3$ H]CP binding to cell surfaces in the presence of lactose (4-O- $\beta$ -D-galactopyranosyl-O-glucose) (Fig. 2, B). Previously, it was shown that simple sugars, glycopeptides, and anti-lectin antibodies can inhibit the cell-cell aggregation (5,17,18).

We next tested the ability of the CP to affect the *in vivo* formation of B16-F1 tumor colonies in the lung. Cells were detached with 2 mM EDTA, suspended in CMF-PBS, and incubated on ice for 30 minutes with CMF-PBS, CP, and MCP. Aliquots of the suspension containing  $10^5$  cells in 0.2 mL PBS were injected intravenously into the tail veins of syngeneic mice. After 17 days, the mice were autopsied, and the number of tumor colonies in the lung were counted (Table 1). A threshold increase in the number of tumor colonies in the lung was observed compared with the control experiment (CMF-PBS alone) when the B16-F1 cells were injected with CP (Table 1) and the effect of CP was dose dependent. To evaluate these findings further, the B16-F1 cells were exposed to and injected with MCP. Incubation of B16-F1 cells with 0.05% MCP resulted in a marked

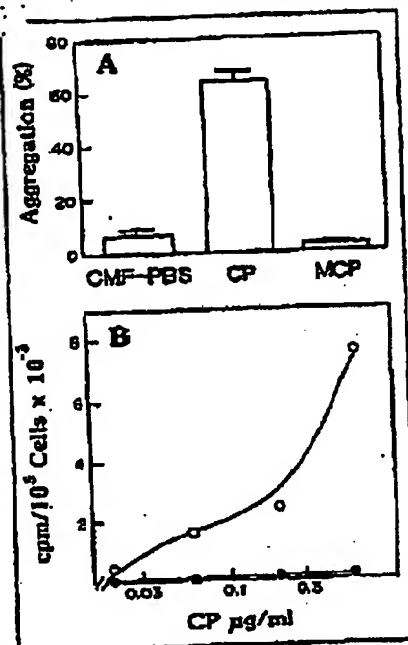


Fig. 2. Binding of CP to B16-F1 melanoma cell surfaces. A) CP-induced homotypic aggregation. Control CMF-PBS. CP—in the presence of unmodified CP (0.05%). MCP—in the presence of modified CP (0.05%). The cells were agitated for 60 minutes at 37 °C, and the degree of cell aggregation was determined as described in the "Materials and Methods" section. B) Binding of CP to B16-F1 cells.  $10^5$  cells were incubated in the presence (●) or absence (○) of lactose (0.15 M) with different concentration of [ $^3$ H]CP (specific activity,  $6.8 \times 10^6$  cpm/mg) for 30 minutes at 4 °C. The cells were washed three times in cold phosphate-buffered saline to remove unbound [ $^3$ H]CP. The cells were then solubilized with 0.1 N NaOH (30 minutes, 37 °C), and the radioactivity was determined in a  $\beta$ -counter. Each point represents the mean of triplicate experiments.

Table 1. Effect of CP and MCP on experimental lung metastasis of B16-F1 melanoma cells

Treatment	No. of mice	Mean No. of lung tumor colonies per mouse (range)
<b>Experiment 1</b>		
CMF-PBS	12	43 (6-136)
CP, $5 \times 10^{-4}$	12	74 (19-102)
CP, $5 \times 10^{-3}$	10	80 (18-120)
CP, $5 \times 10^{-2}$	10	112 (52-112)
CP, $5 \times 10^{-1}$	9	139 (58-172)
<b>Experiment 2</b>		
CMF-PBS	40	33 (10-57)
MCP, $5 \times 10^{-2}$	40	0 (0-1)†
MCP, $5 \times 10^{-4}$	42	0 (0)†

\*Concentration in mol % (w/vol).  
†P < 0.01 from the control (CMF-PBS) (two-tailed, Mann-Whitney U test).

decrease in the ability of these cells to form tumor lung colonization after their intravenous inoculation (Table 1). Fig. 3 shows that treatment with MCP had not only a reduction in the absolute number of experimental metastases but also to an apparent reduction in the volume of the developed metastases. The reason for the change in metastasis volumes observed following treatment with CP and MCP is not clear. It might result from faster or slower retention in the circulation, which may affect the onset of the growth of colonies. The inhibitory effect of MCP was not due to cell toxicity because no effect was observed in their *in vitro* growth properties when the cells were cultured with MCP or CP. Furthermore, injection of  $10^5$  B16-F1 cells at a subcutaneous site in the presence or absence of MCP (0.5%) resulted in the same growth pattern of tumor formation, showing a cytotoxic effect of MCP *in vivo* (not shown).

Several studies using the same B16-F1 melanoma cell system have demonstrated a correlation between the tendency of

cells to undergo intercellular interactions in culture and their metastatic potential [for review see (1)]. *In vivo*, intercellular adhesion by means of cell-surface lectin of one cell and carbohydrate-containing complementary molecules on an adjacent cell or by serum glycoproteins could serve as a bridge between adjacent cells and may contribute to tumor cell embolization resulting with increased organ colonization by the circulating tumor emboli.

The mammalian gal-lectin mediates the recognition process by linking to oligosaccharides with terminal-linked D-galactose residues (19). Investigators also found that somatic mutation, which blocks addition of gal and sialic acid to cellular glycoconjugates, as well as chemical inhibitors of N-linked processing, resulted in an impaired tumor cell adhesion to endothelial cells *in vitro* (20). Other investigators showed that the degree of GlcNAc  $\alpha$ 1-6Man  $\alpha$ 1-6Man  $\alpha$ 1-branching and the completion of these structures with SA $\beta$ 2-3Gal  $\beta$ 1-4 appear to be closely associated with metastatic ability (20-23) and that endothelial cells

may have a lectin with similar specificity where the  $\beta$ 1-4 Gal is part of a larger ligand structure (23,24). Those results indicate that  $\beta$ 1-4 gal-lectin on microvascular endothelial cells can contribute to retention and secondary tumor formation of blood-borne tumor cells. In addition, galactosylation of D36W25 cells (24) increased the number of visible liver metastases after tumor cell injection by 30-fold. The unmodified CP may involve a recognition structure mechanism similar to the D36W25 cell-surface sugar.

The results presented here and in previous studies (3,5) are basically similar to experimental pyelonephritis, whereby infection with *Escherichia coli* can be inhibited by oligomannosides and mannan which bind to the mannose-specific lectins of *E. coli*, and binding of the bacteria to the uroepithelium is prevented (25).

We do not know whether CP and MCP compete with or resemble the yet unidentified natural ligand(s) of the mammalian gal-lectin; however, this study and those described earlier (3-5) may provide a new, simple modality for intervention with the successful colonization of circulating malignant cells.



Fig. 3. Experimental metastasis after intravenous injection of B16-F1 cells ( $1 \times 10^5$ ) without CP (a) or with CP (b) or MCP (c). Eight-week-old female C57BL/6 mice were given an intravenous injection of a 0.3-ml mixture of B16-F1 cells ( $1 \times 10^5$ ) and sugar solution. The mice were killed 17 days after injection, and the tumor colonies per lung were measured under a dissecting microscope.

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## Increasing Incidence of Primary Malignant Brain Tumors: Influence of Diagnostic Methods

Marie Desmeules,\* Tom Mikkelsen, Yang Mao

**Background:** The incidence of brain cancer has increased dramatically over the last decades in most developed countries. Whether these trends can be attributed to improved diagnosis is not clear. **Purpose:** To determine the effect of new imaging technology on increased rates of brain cancer, we assessed the level of detection for neurological disorders when computed tomography (CT) and magnetic resonance imaging (MRI) results were not available. **Methods:** A neurologist performed a blind review of hospital charts from 356 randomly selected patients hospitalized between 1985 and 1989 for neurological disorders, including brain cancer. All prediagnosis information except CT and MRI results was used as a basis for diagnostic re-evaluation. Also, a random sample of 151 brain cancer patients diagnosed between 1960 and 1965 was selected for a description of diagnostic methods used during that period. **Results:** A comparison between the original diagnoses and the re-evaluations for patients in the 1985-1989 sample indicated that there was, among the diseases selected, a 24% misclassification when CT scans and MRI were not available. In particular, 20% of brain tumors were undetected (95% confidence interval = 15%-25%), and 10% of non-tumor disorders were inaccurately labeled as brain tumors in the absence of these tests. The repeatability of the re-evaluations was 86%. **Conclusions:** Among elderly North Americans, at least twofold increases in brain cancer incidence were observed over the last two decades. Since our findings show that CT scans and MRI are responsible for the detection of about 20% of brain tumors, we conclude that

other factors also are responsible for the observed trends. [*J Natl Cancer Inst* 84:442-445, 1992]

Brain cancer is often disabling and fatal. Rates of mortality from brain cancer have increased substantially, especially among the elderly (1-4), over the last decades in most developed countries. Whether such trends reflect a rise in brain cancer risk is controversial. Some investigators have attributed these increasing trends to improved diagnostic methods (5-7). Others argue that because of the magnitude of the increase and because brain cancer rates started to increase before the introduction of new imaging technology, the trends could not be due entirely to improved diagnostic methods (1,2).

Computed tomography (CT), introduced in the 1970s, may partly be responsible for increased tumor detection. Magnetic resonance imaging (MRI), which provides additional anatomic resolution, was introduced in the 1980s. MRI also can increase the rate of detection of tumors, in particular, those in regions of the brain such as the temporal lobe, the brain stem, and posterior fossa that are less easily visualized by other methods.

Numerous studies have assessed the diagnostic value of CT scans and MRI for intracranial disorders (8-18), mainly by comparing their accuracy with other methods of diagnosis. For example, it was found that CT scans had slightly higher sensitivity and specificity compared with radionuclide brain scans (8) and cerebral angiography (9) for the detection of brain tumors and cerebrovascular disease, in particular. The difference in these indices of accuracy between the two tests was only about 3%, however. Other studies indicated that the use of CT scans decreased the perceived need for

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M. Desmeules, Y. Mao, Bureau of Chronic Disease Epidemiology, Laboratory Center for Disease Control, Health and Welfare, Ottawa, Ont., Canada.

T. Mikkelsen, Ludwig Institute for Cancer Research, San Diego, Calif.

\*Correspondence to: Marie Desmeules, M.Sc., Laboratory Center for Disease Control, Tunney's Pasture, LCDC Bldg., Rm. 22C, Ottawa, ON, K1A 0L2, Canada.